MBMG
Metabarcoding & Metagenomics

Metabarcoding and Metagenomics 10: 399–430 (2026)
DOI: 10.3897/mbmg.10.185694

Research Article

Bulk, water, and sediment eDNA metabarcoding reveal metric-driven differences in WFD macrozoobenthos ecological status classification in Slovakia

Michaela Šamulková[1], Pavel Beracko[2], Patrik Macko[2], Kornélia Tuhrinová[1], Zuzana Čiamporová-Zaťovičová[1,2], Fedor Čiampor Jr[1]

1 *Department of Biodiversity and Ecology, Plant Science and Biodiversity Centre, Slovak Academy of Sciences, Dúbravská cesta 9, Bratislava 845 23, Slovakia*
2 *Department of Ecology, Faculty of Natural Sciences, Comenius University in Bratislava, Ilkovičova 6, Bratislava 842 15, Slovakia*
Corresponding author: Patrik Macko (patrik.macko@uniba.sk)

Abstract

Freshwater biomonitoring under the EU Water Framework Directive (WFD) relies on multiple biological quality elements, with macrozoobenthos being one of the most widely used, although still constrained by time-consuming morphological identification and the continued decline in taxonomic expertise. These constraints, together with ongoing rapid biodiversity loss, create an urgent need for high-resolution, scalable assessment methods such as DNA metabarcoding. Here, we evaluated this molecular approach as a WFD-compatible method and compared the performance of bulk and environmental DNA (eDNA) sampling for ecological quality classification in Slovakia. During spring 2022–2023, we sampled 34 WFD long-term monitoring sites using (i) bulk macroinvertebrate kick-samples, and (ii) water and (iii) sediment eDNA. A ~420 bp fragment of the mitochondrial COI gene was amplified using the BF3/BR2 primers, and sequenced. Filtered reads were clustered into OTUs based on 97% identity and taxonomically assigned using the BOLD database. Raw read counts were used as abundance proxies to calculate 12 ASTERICS metrics and multimetric Ecological Quality Ratios (EQRs). Sequencing yielded > 32.1 million reads and 1,205 target OTUs, of which 702 corresponded to 558 Linnaean species. Bulk samples captured the highest richness, followed by water and then sediment eDNA. Bulk samples classified 22 sites as very good Ecological Quality Class (EQC), whereas water and sediment samples frequently reduced EQC by two to three classes. EQC differences were driven mainly by presence/absence metrics (EPT taxa and BMWP), while several abundance-dependent metrics remained stable. Bulk metabarcoding provides a comprehensive signal but still inflates presence/absence metrics, highlighting the need for recalibration and optimised eDNA sampling, including primer selection, to enable comparable non-invasive assessments.

Key words: Molecular approach, multimetric index, water quality

OPEN ACCESS

Academic editor: Florian Leese
Received: 19 January 2026
Accepted: 10 April 2026
Published: 4 May 2026

Citation: Šamulková M, Beracko P, Macko P, Tuhrinová K, Čiamporová-Zaťovičová Z, Čiampor Jr F (2026) Bulk, water, and sediment eDNA metabarcoding reveal metric-driven differences in WFD macrozoobenthos ecological status classification in Slovakia. Metabarcoding and Metagenomics 10: e185694. https://doi.org/10.3897/mbmg.10.185694

Copyright: © Michaela Šamulková et al. This is an open access article distributed under terms of the Creative Commons Attribution License (Attribution 4.0 International – CC BY 4.0).

Introduction

Freshwater ecosystems are increasingly impacted by anthropogenic activities and climate change, with biodiversity declining more rapidly in these ecosystems than in marine or terrestrial environments (Dudgeon 2019; Reid et al. 2019; Albert et al. 2021). This has driven the development of large-scale biomonitoring frameworks, such as the European Union Water Framework Directive (WFD 2000/60/EC), which aims to prevent further ecological degradation and achieve good ecological status of surface waters (EC 2000). Despite extensive monitoring across Europe, only a minority of water bodies currently meet WFD ecological objectives (EEA 2024). Ecological classification is based on biological quality elements (BQEs), including phytoplankton, phytobenthos, macrophytes, macrozoobenthos, and fish, complemented by physico-chemical and hydromorphological parameters (Birk et al. 2012). The assessment follows the "one out, all out" principle, whereby the overall ecological status is determined by the lowest-scoring element. In practice, this means that improvements in individual components, such as macrophytes, have rarely translated into achieving an overall good ecological status, even when other indicators show favourable values (EEA 2024).

Among the BQEs, macrozoobenthos play a key role in aquatic ecosystem functioning and are widely used as sensitive indicators of environmental change (Rosenberg and Resh 1993; Hodkinson and Jackson 2005; Chang et al. 2014; Allan et al. 2021). However, their morphological identification is challenging and increasingly constrained by the declining availability of taxonomic expertise (Wheeler et al. 2004; Löbl et al. 2023). Immature life stages often lack diagnostic characters, leading to frequent identification at higher taxonomic levels, all while the process itself is time-consuming, costly, and prone to observer-dependent error (Haase et al. 2006, 2010; Marshall et al. 2006; Blackman et al. 2019; Chua et al. 2023). These limitations can reduce the reliability and consistency of ecological status assessments, as demonstrated by audits of WFD biomonitoring programmes (Haase et al. 2010; Gleason et al. 2023).

In response to the limitations of morphological identification, recent advances in molecular techniques can offer a promising alternative for biodiversity assessment (Baird and Hajibabaei 2012; Blackman et al. 2019; Liu et al. 2019). DNA metabarcoding enables the simultaneous analysis of multiple taxa and provides a more comprehensive insight into aquatic biodiversity, detecting a broader range of taxa, including hidden diversity (Vivien et al. 2015; Beermann et al. 2018) and facilitating the early detection of rare, protected, or alien invasive species (Comtet et al. 2015; Klymus et al. 2017). This makes it particularly valuable for large-scale biomonitoring programs (Elbrecht et al. 2017; Ficetola and Taberlet 2023). The main advantages of the method are its ability to reduce the time and cost of identification (Hajibabaei et al. 2019; Duarte et al. 2021) and to detect non-native, invasive, or endangered taxa with no additional effort or expense (Fueyo et al. 2024). However, successful identification depends on the completeness of reference databases (Šamulková et al. 2025a), which is steadily improving thanks to numerous national initiatives (GBOL, ABOL, FinBOL, SK-BOL, AquaBOL.SK) aimed at building comprehensive DNA barcode libraries, especially for key groups of bioindicators (Szucsich 2015; Morinière et

al. 2017; Roslin et al. 2022; Macko et al. 2024). Although many studies highlight the potential of DNA metabarcoding, it has not yet been routinely implemented in biomonitoring under the WFD, partly because of the absence of explicit abundance data, along with other technical and standardisation-related limitations, such as primer bias, unequal amplification efficiencies across taxa, incomplete reference databases, and variability in laboratory protocols (Elbrecht and Leese 2015). In addition to these technical and methodological challenges, legislative and regulatory constraints play a significant role in limiting the adoption of DNA-based approaches within WFD-compliant biomonitoring (Hering et al. 2010). Abundance data are particularly important in countries such as Germany and Slovakia, where ecological status is evaluated using multimetric indices that rely on abundance-dependent metrics (e.g., Saprobic Index, Rheoindex). One proposed solution is to transform DNA data into a presence/absence format (i.e., replacing abundance values with binary data: 1/0; Buchner et al. 2019), or alternatively, to use read counts per taxon as potential proxies for abundance (Deagle et al. 2019; Di Muri et al. 2020; Salis et al. 2024). However, the use of read counts as a proxy for abundance has several limitations, as read numbers can be influenced by biases introduced during PCR amplification and primer specificity (Doi et al. 2017; Krehenwinkel et al. 2017; Shelton et al. 2023), as well as by differences in organism biomass, which may lead to unequal representation of taxa and reduced detection of small or rare species (for synopsis, see Elbrecht et al. 2021). Nevertheless, the aforementioned studies (e.g., Salis et al. 2024) suggest that read counts may still provide ecologically relevant information when interpreted with appropriate caution. Šamulková et al. (2025b) also used read counts to calculate a multimetric index and their results showed that most variation occurred in metrics based on presence/absence (e.g., BMWP Score, Number of Families, EPT Taxa), while abundance-dependent metrics (e.g., Saprobic Index, Index of Biocoenotic Region, Rhithron Type Index) remained relatively stable. These findings suggest that, if appropriately interpreted, read data may support the broader application of DNA-based approaches in ecological status assessments. Despite these promising applications, DNA metabarcoding is still subject to technical limitations, including false positives, incomplete or inconsistent reference databases, and sequencing or amplification biases (Schultz and Hebert 2022; Baena-Bejarano et al. 2023; Fueyo 2024; Šamulková et al. 2025a, 2025b).

To enable DNA metabarcoding to become part of routine monitoring under the WFD, it is also crucial to select the appropriate sampling methods. Given the focus of monitoring on the aquatic environment, different types of samples can be analysed. These include bulk samples and environmental DNA (eDNA) from water or sediment. Bulk samples are collected using traditional "kicking techniques" (e.g., according to Frost et al. 1971) and contain benthic invertebrates along with organic and inorganic material. However, the sampling process is significantly influenced by the chosen methodology and can be problematic, especially at high flow rates in larger rivers (Bonada et al. 2006; Buss et al. 2015; Doretto et al. 2020). Bulk samples also require a considerable amount of fixative (usually more than 0.5 L of 96% ethanol) and are bulky for storage. Their most notable disadvantage, however, is the invasiveness of the method (Kirse et al. 2022), which can be particularly problematic

in smaller streams. On the other hand, the advantage of bulk samples is the exact localization of the sampling site, which allows for reliable determination of the species composition present at the time of sampling. Bulk samples also provide DNA predominantly from target and commonly used invertebrate bioindicator taxa, thereby ensuring high relevance for ecological assessment (Macher et al. 2018). Environmental DNA samples are obtained by filtering water through special filters (Ficetola et al. 2008) or by collecting benthic sediment (Pawlowski et al. 2022). They are non-invasive, require less fixative, and take up less space for storage, with relatively fast processing times (V. den Bulcke et al. 2024). A methodological limitation includes the collection of water containing upstream DNA, which may lead to the detection of species that were not actually present at the monitoring site (Fonseca et al. 2023). Others include difficulties in sediment selection and collection due to substrate composition and water levels (Pawlowski et al. 2022). A newly proposed method, capture-incubate-release (CIR) metabarcoding (Sander et al. 2025a), could potentially be used as an intermediate approach between the bulk sampling and eDNA-based methods from water or sediment. Their study demonstrated that most locally occurring species can be reliably detected via eDNA after short-term incubation in water, allowing organisms to be subsequently released back into their natural environment. This method enhances taxon detectability while maintaining a non-invasive character and may thus represent a practical and ecologically sound compromise between invasive bulk sampling and conventional eDNA-based approaches.

Despite the undeniable potential of DNA-based approaches, further empirical testing is required before their widespread adoption in routine ecological monitoring. In this study, we evaluate the efficiency and applicability of DNA metabarcoding for the assessment of the ecological status of surface waters in accordance with the WFD, using bulk and eDNA water and sediment samples collected from long-term monitoring sites in the Western Carpathians in Slovakia. The dataset complements previous work (Šamulková et al. 2025b), which, although based on the same sample types (bulk, water eDNA, and sediment eDNA), focused on the comparison between conventional (morphology-based) and DNA-based approaches using a smaller number of sampling sites. In contrast, the present study is based on an expanded dataset, thereby allowing a broader assessment of DNA metabarcoding across different sample types. Our main objectives were to compare the taxonomic composition among these sample types and to assess their impact on the resulting Ecological Quality Ratio (EQR) values and individual ecological metrics within the multimetric index applied in both the EU and Slovak assessment schemes. To the best of our knowledge, this study represents one of the first applications using two types of eDNA samples to calculate such a broad range of ecological metrics and the overall multimetric index. Given that bulk samples typically capture higher taxonomic richness, we hypothesized that this would influence ecological status classification. Furthermore, we hypothesized that bulk samples would yield more consistent and ecologically comparable results to water samples, whereas sediment samples would produce more variable or potentially biased assessments due to lower taxonomic resolution and the accumulation of eDNA.

Materials and methods

Data collection

The material was collected from 34 WFD monitoring sites, representing 10 stream types, according to Šporka et al. (2009), ranging from 130 to 905 m a.s.l. across Slovakia in 2022 or 2023 during the spring season (Fig. 1, Suppl. material 1). At each site, three types of samples were collected: (1) macrozoobenthos bulk samples obtained following Frost et al.'s (1971) kick-sampling technique, with a ~15 min duration, covering all major microhabitat types proportionally to their occurrence at each site; (2) two replicate water samples collected along a transverse transect across the stream by filtering 1 litre through cellulose filters (0.45 µm pore size), targeting the water column while avoiding sediment disturbance; and (3) two 25 mL replicate sediment samples collected from different microhabitats (riffle and pool sections). All samples were preserved in 96% ethanol and stored at -25 °C. For more detailed information on the collection of individual sample types, see Šamulková et al. (2025b).

Laboratory processing

Sample processing followed the methodology described by Šamulková et al. (2025b). For all sample types, DNA was isolated and purified using the ReliaPrep gDNA Tissue Miniprep System (Promega) or DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol. Briefly, DNA from water samples was extracted from dried and torn cellulose filters using the isolation kit. DNA from sediment samples was isolated using a combination of phosphate buffer and the isolation kit following Taberlet et al. (2012). Briefly, sediment samples preserved in ethanol were first dried at 55 °C for 3 hours to remove the fixative and then mixed with phosphate buffer in a 1:1 ratio. Samples were homogenised using a rotator and subsequently centrifuged at 4,000 rpm for 30 minutes. The resulting supernatant was filtered through cellulose syringe filters (0.45 µm pore size), which were then dried, torn into pieces, and subjected to DNA purification using the same kit. For bulk samples, non-target material and inorganic matter were removed during a 90-minute pre-sorting step. The entire sample, including the fixative, was then homogenised for three minutes using a kitchen blender, and DNA was extracted in three replicates of 1 mL using the kit. Purified genomic DNA was stored at -25 °C.

Amplification of the target fragment (~420 bp within the standard COI barcode region) was performed using a two-step PCR approach (Elbrecht and Steinke 2019). Each DNA extract was amplified into two PCR replicates. Ten negative controls were randomly distributed across each 96-well plate to monitor potential contamination. The first amplification used BF3 and BR2 primers (Elbrecht et al. 2019), followed by a second amplification with the same primers with adapters and indices for Illumina MiSeq sequencing. PCR success was verified via 1% agarose gel electrophoresis, and product concentrations were assessed using the Carestream MI Application. Equimolar pooled libraries were prepared, purified using the Wizard SV Gel and PCR Clean-Up System (Promega), and sequenced on an Illumina MiSeq (Reagent Kit v3, 2 × 300 bp) at the Institute of Chemistry, Slovak Academy of Sciences.

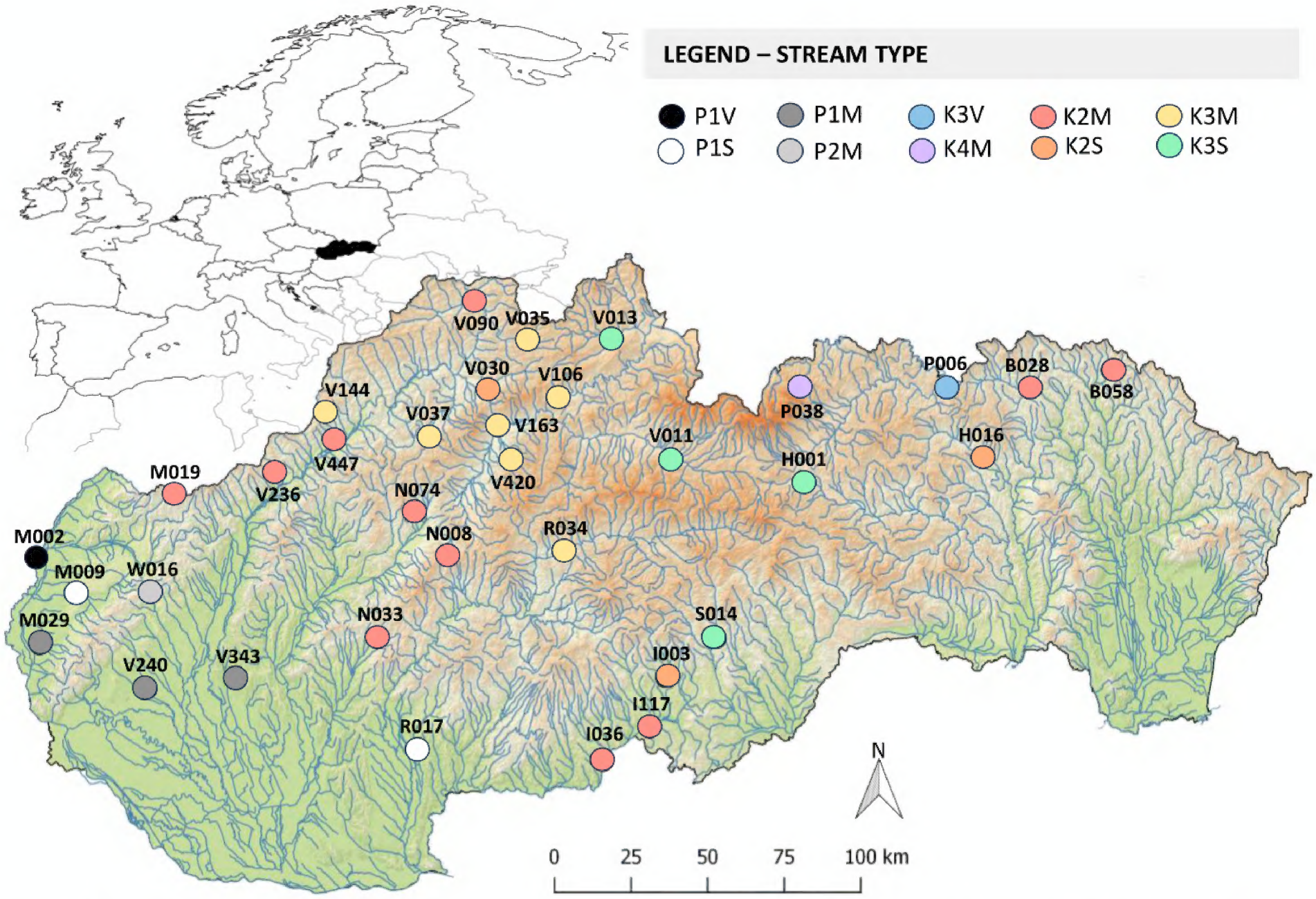

Figure 1. Geographical distribution of the sampling sites across Slovakia, with stream types marked by coloured circles. Ecoregions are classified as Pannonian (P) and Carpathian (K). Altitude categories are defined as: 1 (<200 m a.s.l.), 2 (200–500 m a.s.l.), 3 (500–800 m a.s.l.), and 4 (>800 m a.s.l.). Flow size categories include small (S), medium (M), and large (V). The map was created using QGIS version 2.18.15.

Data processing and analyses

Bioinformatic processing of sequencing data followed the approach outlined in Vargovčík et al. (2024), with modifications based on updated software versions. Briefly, raw sequencing reads were demultiplexed, trimmed to remove primers (Cutadapt v5.0, Martin 2011), and merged (PEAR v0.9.11, Zhang et al. 2013). Subsequently, they were processed through dereplication, denoising, and length filtering (Vsearch v2.15.1, Rognes et al. 2016). Translation-based filtering was applied post-denoising (metaMATE v0.2b6, Andújar et al. 2021). Then, chimera removal and greedy OTU clustering at a 97% identity threshold were performed (Vsearch v2.15.1, Rognes et al. 2016). This step was followed by default post-clustering curation (LULU v0.1.0, Frøslev et al. 2017). Taxonomic assignment was performed using BOLD (Ratnasingham and Hebert 2007) via BOLDigger3 v1.4.0 (Buchner and Leese 2020), using similarity thresholds of 99% for species-level assignments and 97% for higher taxonomic levels. OTUs with > 85% similarity to the best database match were retained, focusing on target animal phyla (Annelida, Arthropoda, Bryozoa, Cnidaria, Chordata, Mollusca, Nematoda, Nematomorpha, Platyhelminthes, Porifera, and Rotifera). To account for potential contamination, negative controls were evaluated separately for each sequencing run. For OTUs detected in negative controls, the maximum

read count was identified and subtracted from all samples within the respective run. This approach reduced the influence of contamination without removing entire OTUs from the dataset. Following this filtering, only taxonomic groups used in routine biomonitoring (Insecta, Gastropoda, Bivalvia, Amphipoda, Isopoda, Mysidacea, Polychaeta, Oligochaeta, Hirudinea, and Turbellaria) were included in the calculation of ecological status (hereafter referred to solely as ES).

The dataset containing these groups was further formatted according to the requirements of TaxonTableTools v1.5.1 (Macher et al. 2021), where samples were merged by location for each sample type (bulk, water, and sediment). Records with fewer than 10 reads per sample were removed to reduce potential noise (Suppl. material 2). Sequencing depth was summarised as read counts per locality for each sample type and visualised using log10-transformed values. The resulting filtered dataset was adapted to the input format required by the ASTERICS v4.04 (Furse et al. 2006), where individual ecological metrics were calculated. Sequencing read counts were used as proxy variables for abundance data, following the explicit implementation of this approach in Šamulková et al. (2025b). In cases where a single species was represented by multiple OTUs, read counts were counted and assigned to a single Linnaean taxon. The selection of metrics for calculating EQRs and the resulting ES classification was guided by stream typology, with 10 stream types and 12 different metrics used following the framework established by Šporka et al. (2009) (Suppl. material 3). Using the metric values obtained from ASTERICS, partial EQR values were calculated using reference values (Suppl. material 4). The final multimetric EQR value for each site was calculated as the arithmetic mean of the partial EQR values derived from individual metrics and was subsequently used to assign the ecological quality class. Additionally, multimetric EQR values were recalculated with selected presence/absence metrics excluded, specifically those exhibiting significant differences between bulk and environmental samples, to assess their influence on the resulting ES classification.

Prior to all performed analyses, the distribution of all compared data was tested for normality using the Shapiro–Wilk test at a significance level of 0.05 and homogeneity of variances was assessed using Bartlett's test. If the data were consistent with a normal distribution, differences in the values of the multimetric EQR and individual metrics among the compared sample types were tested using one-way ANOVA, followed by post-hoc comparisons with the Tukey HSD method. If the data did not reflect a normal distribution, differences in the values of individual metrics among the compared sample types were tested using the Kruskal–Wallis test, followed by non-parametric post-hoc comparisons based on the Chi-squared statistic. Differences between two related samples obtained from the compared sampling methods were analysed using the paired t-test, which evaluates whether the mean difference is significantly different from zero, assuming normal distribution of the differences. When the assumption of normality was violated, the Wilcoxon signed-rank test was applied as a non-parametric alternative to the paired t-test, assessing whether the median of the differences significantly deviates from zero. All analyses and plots were made in R 4.2.1 (R Core Development Team 2022) using the "stats" (R Core Development Team 2022), "pgirmess" (Giraudoux 2023), and "beanplot" (Kampstra 2008) packages.

Results

Sequencing output and taxonomic composition

Sequencing of all samples (bulk, water eDNA, and sediment eDNA) yielded over 32.1 million reads across 34 sampling sites. After quality filtering, more than 16 million reads were retained, of which over 7 million reads originated from bulk samples, 3.6 million from water samples, and nearly 5 million from sediment samples. These reads were subsequently clustered into 24,529 OTUs, of which more than 21,000 were removed due to low sequence similarity (<85%) or detection in negative controls. Subsequent filtering of non-target taxa (e.g., algae, diatoms, chordates, terrestrial organisms, and benthic invertebrates nonrelevant for biomonitoring) resulted in 1,205 OTUs. Identification to species was possible for 702 OTUs, corresponding to 558 distinct Linnaean species. This discrepancy indicates the presence of potential cryptic diversity (hereafter PCD), where multiple OTUs were assigned to a single Linnaean species. The majority of OTUs belonged to Insecta (544 OTUs = 463 spp.) and Clitellata (115 OTUs = 66 spp.). Among insects, the most species-rich orders were Diptera (224 spp.), Trichoptera (86 spp.), Ephemeroptera (59 spp.), Coleoptera (38 spp.), and Plecoptera (37 spp.). PCD was observed in 94 species, represented by a total of 235 distinct OTUs. The highest levels of PCD were found in the families Chironomidae (24 species = 51 OTUs) and Naididae (13 species = 48 OTUs). The most OTU-rich species were *Tubifex tubifex* (Müller, 1774) with 11 OTUs, *Limnodrilus hoffmeisteri* Claparède, 1862 with 10 OTUs, and several taxa represented by five OTUs each (e.g., *Baetis rhodani* (Pictet, 1843), *Stylodrilus heringianus* Claparède, 1862, *Gammarus fossarum* Koch, 1836).

Taxonomic richness across sample types

The spectrum of detected taxa varied among sample types and was also influenced by data processing procedures (Suppl. material 5), as well as by differences in sequencing depth. These differences were reflected in read counts per locality (Suppl. material 6), with bulk samples consistently yielding higher values than sediment and water eDNA. When PCD was retained, bulk samples exhibited the highest taxonomic richness, with 895 OTUs, of which 610 were identified to species level. Water samples yielded 680 OTUs (414 spp.), sediment samples showed the lowest richness (430 OTUs, 234 spp.). After removing PCD (multiple OTUs assigned to a single Linnaean species), bulk samples still captured the highest taxonomic richness (599 unique taxa, 486 spp.), water samples contained 417 taxa (344 spp.), and sediment samples included 233 taxa (183 spp.).

The dataset adjusted for analysis in the ASTERICS, i.e. matching the taxon spectrum accepted by the program, included 576 taxa (470 identified to species) for bulk, 404 taxa (334 identified to species) for water, and 222 taxa (181 assigned to species) for sediment samples.

Ecological status and multimetric EQR across sample types

The ES assessment, based on macrozoobenthos and environmental DNA metabarcoding, revealed pronounced variability among sample types (the recalculated partial and multimetric EQR values for each site and sample

type are provided in Suppl. material 7). Bulk samples classified 22 sites as having a very good (1) ES, whereas water and sediment samples often resulted in a worse ecological classification of these sites, by up to 2 or 3 classes (Suppl. material 7). In general, sediment samples tended to produce poorer ecological assessments, with 24 sites assigned to class 3 (moderate) or 4 (poor). Only three sites (I036, N074, P038) showed consistent ES across all sample types. When data from all three sample types were pooled ("All samples"), most sites (82%) were classified into the highest ecological quality class (EQC; very good status).

Values of the multimetric EQR differed significantly among sample types in both comparisons (Table 1). Follow-up post-hoc tests identified significant differences between eDNA (water and sediment) samples and the other two types (all samples, bulk) in the all samples × bulk × eDNA comparison. In the comparison of bulk × water × sediment samples, post-hoc tests revealed significant differences among all pairs (Fig. 2A). In paired tests, the mean or median of the differences significantly differed from zero in all pairs of compared sample types (Fig. 2B). In the paired comparison between all samples and bulk samples, the mean difference was approximately five times lower than the mean difference in the comparison between all samples and eDNA samples. In the paired comparison of bulk samples × water samples and bulk samples × sediment samples, the means and distribution of differences were very similar, although slightly lower differences were observed for sediment samples. Most of these differences were positive, indicating that bulk samples generally yielded higher EQR values in both comparisons.

Table 1. Results of parametric (one-way ANOVA) and non-parametric (Kruskal–Wallis) tests comparing EQR values among sample types for the multimetric index as well as for individual metrics. The ANOVA section includes degrees of freedom (between-group and within-group values), F-statistic, and p-values. The Kruskal–Wallis section reports the Chi-squared statistic, degreez of freedom, and p-value.

	F-statistic	Degrees of freedom	*p*-value
Ecological quality ratio – EQR (Multimetric index)			
All samples vs. Bulk samples vs. eDNA samples	16.41	2, 99	**< 0.0001**
Bulk vs. Water vs. Sediment	36.39	2, 99	**< 0.0001**
Ecological quality ratio – EQR (Bulk vs. Water vs. Sediment)			
Saprobic index	4.96	2, 99	**< 0.01**
[%] Oligosaprobic taxa	1.56	2, 99	0.215
[%] Type Aka+Lit+Psa (scored taxa = 100%)	2.55	3, 99	0.083
	Chi-squared statistic	**Degrees of freedom**	**p-value**
Individual ecological metrics			
BMWP score	63.01	2	**< 0.0001**
Rhithron Type Index	8.21	2	**< 0.05**
EPT taxa	60.962	2	**< 0.0001**
Index of Biocoenotic Region	16.75	2	**< 0.001**
Rheoindex	1.33	2	0.52
[%] metarhithral (scored taxa = 100%)	13.1	2	**< 0.01**
Margalef index	15.41	2	**< 0.001**
[%] Gatherers/Collectors (scored taxa = 100%)	14.09	2	**< 0.001**
Number of Families	17.59	2	**< 0.001**

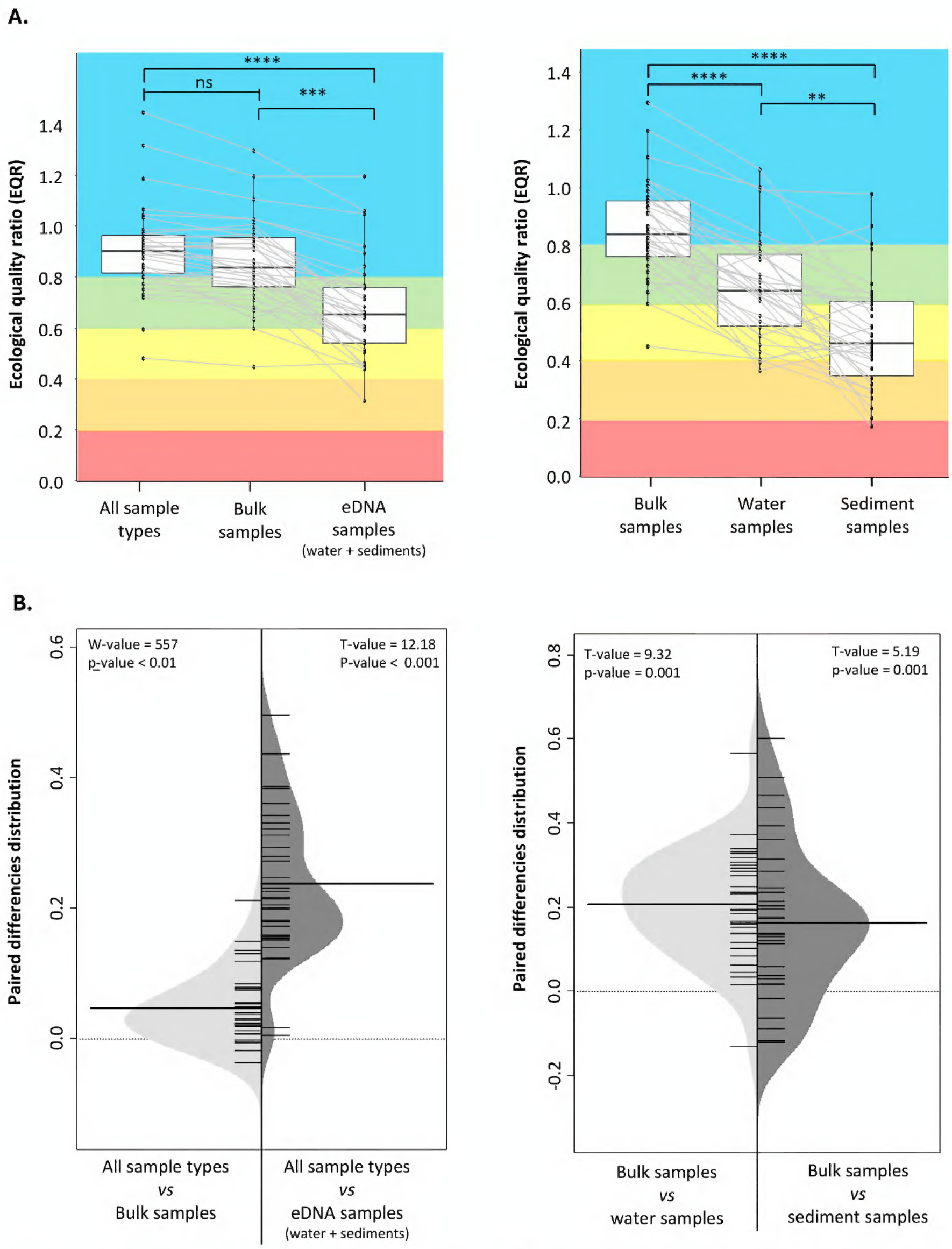

Figure 2. Comparison of multimetric EQR values among sample types. Boxplots showing differences in EQR values between sample types, including results of Tukey HSD post-hoc tests. Box boundaries represent the 25th and 75th percentiles, the central line indicates the median, and points represent individual EQR values for each site. Coloured background bands indicate ecological quality classes (EQC): red – bad, orange – poor, yellow – moderate, green – good, and blue – very good. Beanplots showing paired differences in EQR values between sample types, together with results of paired statistical tests (paired t-test or Wilcoxon signed-rank test). Short horizontal lines represent individual paired differences, while the long horizontal line indicates the mean or median difference. Significance levels: **** p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05; ns – not significant.

Performance of individual ecological metrics

For individual metrics, EQR values did not differ significantly for three out of twelve indicators (Percentage of oligosaprobic taxa, Akal+Lital+Psamal taxa, Rheoindex – Fig. 3) across the three sampling methods. Post-hoc analyses revealed significant differences in six metrics (Saprobic Index, Rhithron Type Index, Index of Biocoenotic Region, Margalef Index, Percentage of metarhithral taxa, Gatherer/collector taxa) between bulk and sediment samples. Significant differences between water and sediment samples were found for only two of these parameters (Index of Biocoenotic Region, Percentage of metarhithral taxa). Significant differences between bulk and water samples were detected in three metrics (Number of Families, EPT taxa, BMWP score). The Number of Families metric differed significantly between bulk and eDNA samples, and between water and sediment samples. EPT taxa and BMWP scores showed significant differences across all sample-type comparisons. The EPT taxa and BMWP index values differed substantially given the varying number of taxa detected across sample types (Fig. 4). Overall, bulk samples yielded the highest values in both metrics, detecting 62 unique EPT taxa (Ephemeroptera, Plecoptera - 11 spp., Trichoptera - 40 spp.) and 18 BMWP families with scores ranging from 1 to 10. Water and sediment samples yielded considerably lower values, detecting only six unique EPT taxa in total, with three BMWP families in water samples and two in sediment samples. Detailed lists of taxa (EPT taxa and families contributing to the BMWP score and Number of Families metrics) are provided in Suppl. material 8: 8a, 8b, allowing for a more comprehensive overview of the taxonomic composition underlying these metrics.

Finally, recalculation of multimetric EQR values after omitting the three metrics that showed significant differences between bulk and environmental samples (EPT taxa, BMWP score, and Number of Families) resulted in reduced and overall attenuated differences among sample types (Suppl. material 9). In the comparison of bulk × water × sediment samples, differences between bulk and water samples were no longer statistically significant, while differences involving sediment samples remained less pronounced. Similarly, in the comparison of bulk/all samples × eDNA samples, differences were reduced.

Discussion

Our results suggest that ES classification under the WFD may be strongly influenced by the choice of sample type. However, these differences likely reflect not only underlying ecological patterns but also properties of the applied methodological approaches. Bulk samples generally exhibited higher taxonomic richness and tended to indicate better ES compared to water and sediment eDNA, which more often resulted in lower EQC values. These differences were primarily associated with presence/absence metrics, while abundance-dependent metrics remained relatively stable, highlighting the sensitivity of current assessment frameworks to taxon detection. After removing metrics based on presence/absence data, namely EPT taxa, BMWP score, and Number of Families, these differences were markedly reduced, with bulk and water samples often yielding comparable EQC values, suggesting their strong potential for future implementation within the WFD framework.

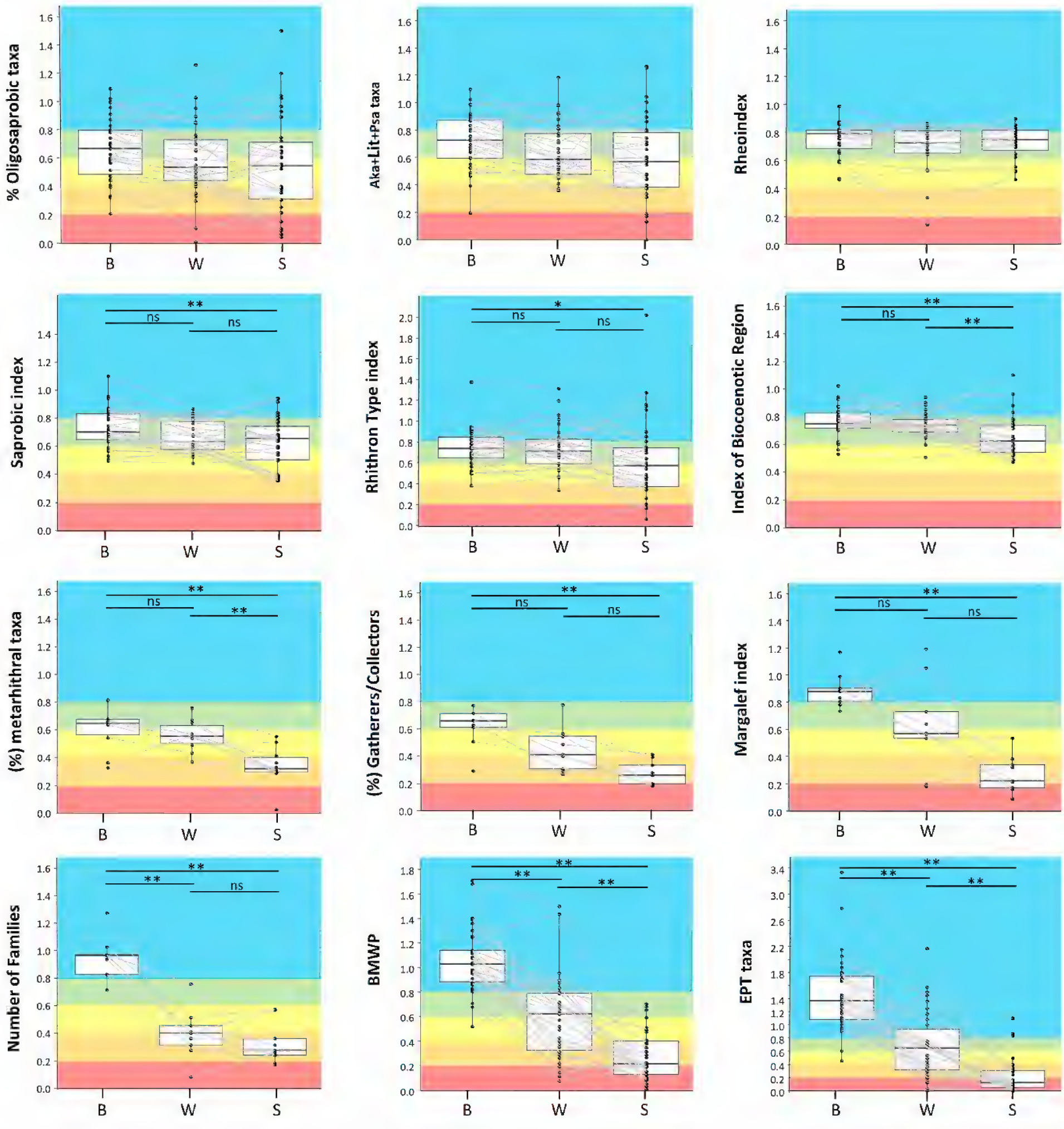

Figure 3. Comparison of EQR values for individual ecological metrics across sample types. Boxplots show differences in metric-specific EQR values between bulk (B), water (W), and sediment (S) samples, together with results of Tukey HSD post-hoc tests or non-parametric post-hoc comparisons. Box boundaries represent the 25th and 75th percentiles, the central line indicates the median, and points represent individual site values. Coloured background bands indicate ecological quality classes (EQC): red – bad, orange – poor, yellow – moderate, green – good, and blue – very good. Significance levels: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; ns – not significant.

In this context and given that WFD objectives remain unmet across most EU countries (EEA 2024), these findings further highlight the need for more reliable and efficient monitoring approaches. DNA metabarcoding represents a highly promising tool in this regard, offering increased taxonomic resolution and scalability. At the same time, some of the differences observed among sample types may reflect methodological aspects, such as primer performance, where

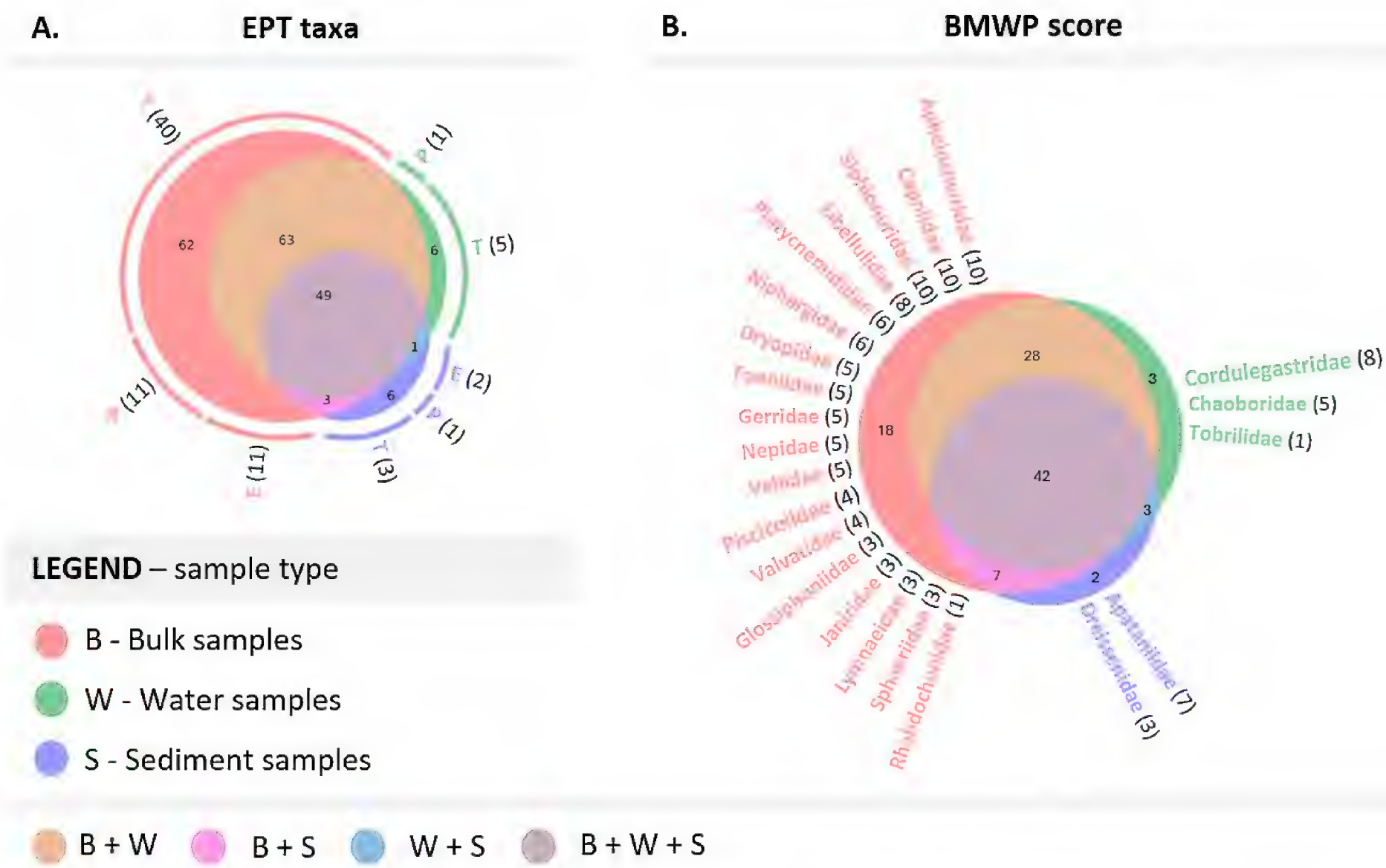

Figure 4. Overlap of taxa detected across three sample types, bulk samples (red), water eDNA (green), and sediment eDNA (purple), for two ecological metrics. (A) Venn diagram of EPT taxa, where letters denote Ephemeroptera (E), Plecoptera (P), and Trichoptera (T); numbers in parentheses indicate the total number of taxa detected per group. Values within the diagram represent taxa unique to or shared among sample types. The complete list of EPT taxa is provided in Suppl. material 8: 8a. (B) Venn diagram of BMWP families, with family names and corresponding BMWP scores displayed around the diagram and colour-coded by sample type. The complete list of families with BMWP scores is provided in Suppl. material 8: 8b. Venn diagrams were generated using TaxonTableTools (TTT).

the use of more eDNA-optimised primer sets could improve comparability with bulk samples (Leese et al. 2021; Brantschen et al. 2022). Recent WFD-oriented studies further support both the strong potential and current challenges of metabarcoding (Macher et al. 2025; Fueyo et al. 2025; Sander et al. 2025b; van der Lee et al. 2026). For example, intercalibration within the German WFD framework showed ~70% agreement between DNA-based and morphology-based classifications, while also highlighting the need for methodological harmonisation (Macher et al. 2025). A recent review by Fueyo et al. (2025) further emphasises key challenges, including detection uncertainties and incomplete reference databases, as well as the need to adapt existing indices to molecular data. Together, these findings indicate that while DNA metabarcoding is already a powerful approach, its full integration into WFD monitoring will benefit from continued methodological standardisation and optimisation.

Performance of different sample types in DNA-based biomonitoring

This study evaluated the use of three sample types - bulk and water and sediment eDNA samples in DNA metabarcoding for freshwater macrozoobenthos. Across 34 WFD monitoring sites, we detected 1,205 OTUs belonging to taxonomic groups targeted by conventional monitoring methods. However, the

results revealed clear differences in taxonomic resolution among the sample types. Sediment samples recovered only 35.68% of the total OTUs, water samples 56.43%, and bulk samples showed the highest richness, detecting 74.27% of all OTUs. The high efficiency of bulk samples is consistent with findings from previous studies, such as Gleason et al. (2021), Múrria et al. (2024), or Derycke et al. (2021). Macher et al. (2018) also compared taxonomic composition between bulk and water samples, finding that although water samples yielded a higher total number of taxa, the target bioindicator groups, such as Ephemeroptera, Plecoptera, and Trichoptera (EPT), were more abundant in bulk samples. One possible explanation for our results may be the use of the BF3/BR2 primer pair with a relatively long amplicon length (~420 bp), which can hinder the amplification of degraded extracellular DNA typical of eDNA samples (Vourka et al. 2023). This may have contributed to the lower number of taxa detected in our eDNA samples, which also exhibited markedly lower sequencing depth compared to bulk samples. Although BF3/BR2 primers are often considered suboptimal for eDNA applications (e.g., Macher et al. 2018; Leese et al. 2021; Rivera et al. 2021), Majaneva et al. (2024) showed that they did not lead to excessive amplification of non-target OTUs in water samples. This suggests that primer performance may vary depending on sample type (i.e. abundance of non-target taxa) and associated environmental or technical factors that are not yet fully understood. In general, primers targeting shorter fragments are considered more suitable for eDNA, as they improve the amplification of degraded DNA (for a comprehensive overview of primer sets, see Vamos et al. 2017). For instance, the fwhF2/EPTDr2n primer pair (~142 bp; Leese et al. 2021) was specifically developed to enhance the detection of freshwater invertebrates and has been shown to increase the recovery of indicator taxa, particularly within EPT families (Brantschen et al. 2022).

Nevertheless, most comparative studies indicate that differences between sample types are not solely driven by primer choice but also reflect inherent differences in the nature of the samples themselves. Different sample types tend to capture distinct taxonomic subsets, resulting in low species-level overlap (Múrria et al. 2024; van der Lee et al. 2026). This is consistent with the findings of Sander et al. (2025b), who showed that differences between bulk and eDNA samples often reflect differences in the type of ecological signal captured (local vs. spatially integrated), rather than methodological bias alone. This is one of the main reasons why a combination of bulk and water samples is often recommended, with bulk samples primarily capturing benthic fauna and water eDNA detecting organisms present in the water column (Múrria et al. 2024).

Potential cryptic diversity and implications for ecological assessment

Beyond differences in taxonomic richness among sample types, our results also highlight an important and often overlooked aspect of biodiversity assessment; substantial genetic variation within morphologically defined taxa, commonly referred to as cryptic diversity (Hending 2025). In this study, however, we use the term potential cryptic diversity (PCD), as these patterns are inferred from sequence divergence and clustering thresholds rather than independently validated species boundaries. In freshwater macroinvertebrates, intraspecific COI divergence can exceed commonly applied clustering thresholds due to various

evolutionary and population processes (e.g., phylogeographic structure or historical isolation), which may result in multiple OTUs being assigned to a single Linnaean species (e.g., Morinière et al. 2017; Raupach et al. 2022; Zhang and Bu 2022; Macko et al. 2024, 2025). In our dataset, PCD was particularly evident in Chironomidae and Naididae, as well as in widespread species such as *Tubifex tubifex* (11 OTUs) and *Limnodrilus hoffmeisteri* (10 OTUs), whose cryptic diversity was already documented by several studies (e.g., Pfenninger et al. 2007; Vivien et al. 2020; Mrozińska and Obolewski 2024). In general, morphological identification of oligochaetes is challenging or even impossible without mature individuals and dissection. The *T. tubifex* complex is known to consist of multiple lineages with distinct biological and ecological traits, and together with *L. hoffmeisteri*, urgently requires taxonomic revision (Erséus and Gustafsson 2009). Another group characterized by notable cryptic diversity are chironomids (Beermann et al. 2018). They represent one of the most OTU-rich insect groups in our dataset (277 OTUs), including 133 Linnaean species assigned to 160 OTUs. The metabarcoding analysis of Andújar et al. (2017) even resolved a single chironomid morphotaxon into 55 OTUs, highlighting both the extent of hidden diversity and the resolving ability of DNA-based methods. Chironomid larvae often comprise around 50% of macrozoobenthos abundance (Pinder 1986; Armitage et al. 1995), yet their identification remains highly demanding. Due to their abundance and the difficulty of accurate morphological determination, chironomids are often used in ecological assessments only at the family or subfamily level (Ferrington et al. 1991; Beermann et al. 2018). We argue that molecular tools offer a promising solution for more precise identification, which may allow for the development of autecological characteristics at lower taxonomic levels.

The composition of detected taxa also varied depending on how PCD was processed. When multiple lineages were merged into a single morphologically defined Linnaean species, the differences in taxa richness between sample types decreased, but bulk samples still showed the highest richness. Despite the high species-level coverage achieved through DNA metabarcoding, only a portion of the detected taxa could be used for ES assessment. This is because the ASTERICS program operates with a reference list of approximately 9,550 taxa at various taxonomic levels, but not all of them contain autecological characteristics (Hering et al. 2004). This list does not account for cryptic diversity and excludes many taxa that are difficult to identify morphologically or lack assigned autecological characteristics. Of the nearly 900 OTUs detected in bulk samples, only 576 taxa (~ 65%) were included in ASTERICS. This proportion was 59.4% for water samples and 52% for sediment samples. This discrepancy highlights one of the main challenges in implementing DNA metabarcoding in routine biomonitoring: the need to expand the database in ASTERICS with autecological information corresponding to the taxonomic resolution provided by molecular methods. In response to this issue, researchers in New Zealand (Wilkinson et al. 2024) have already developed a Taxon-Independent Community Index (TICI) based on ASV data, which translates complex metabarcoding outputs into interpretable ecological metrics and allows for ES assessments without relying on traditional morphological taxonomies. These findings illustrate that the current limitations of DNA-based ecological assessment are largely methodological and conceptual, rather than technical, and are primarily linked to the structure of existing assessment frameworks.

Drivers of multimetric EQR and metric sensitivity

Building on the observed differences in taxonomic composition and metric input data, we next consider how these patterns translate into ES assessment. Results based on the metabarcoding data show that the choice of sample type affects the final classification into EQCs. While the combined data from all three sample types classified most of sites into the highest EQC (very good ES), individual water and sediment samples often resulted in a lower classification - by up to 2 or 3 classes. This trend is consistent with the findings of Múrria et al. (2024), who observed that bulk samples tend to produce more favourable ES assessments compared to eDNA from water, while also capturing a broader taxonomic spectrum. However, our more detailed comparison of the three sample types revealed that, although the overall distribution of multimetric EQR values is more similar between bulk and water samples than between bulk and sediment samples, paired comparisons of individual sites show slightly greater similarity between bulk and sediment samples. This indicates that, among the eDNA samples, sediment samples may, in some cases, provide a more consistent approximation of bulk-sample-based assessments than water eDNA, likely because they better reflect local benthic communities (Gleason et al. 2021; Wang et al. 2021). Ji et al. (2022) further showed that sediment eDNA metabarcoding can yield higher detected species richness compared to traditional morphological approaches. However, this pattern should be interpreted with caution, as eDNA can accumulate and persist in sediments, potentially reflecting not only the current community but also historical signals (Turner et al. 2015). In contrast, the lower similarity between bulk and water samples may be largely driven by downstream transport of eDNA from upstream parts of the catchment (Fonseca et al. 2023), as well as by temporal and environmental factors affecting eDNA persistence and detectability, such as flow conditions, temperature, or UV exposure (Joseph et al. 2022). Previous studies comparing conventional approaches with DNA metabarcoding using a multimetric index have predominantly relied on bulk samples (e.g., Macher et al. 2025; Šamulková et al. 2025b). The studies demonstrate that ES assessment from bulk samples has led to an improvement in the EQC at 26% of sites in Germany (Macher et al. 2025) and 60% of sites in Slovakia (Šamulková et al. 2025b). In a Spanish study applying the IBMWP index, both bulk and water samples were compared to the conventional method (Múrria et al. 2024). Water samples resulted in an improved ES at one out of five sites (20%), while bulk samples showed improvement at two sites (40%). Applying the same comparison framework to our water eDNA data would result in a change in EQC at only 19% of sites, further highlighting the reduced sensitivity of water-based eDNA for ES classification under the current assessment scheme.

To better understand which components of the multimetric index drive differences in ES among sample types, we examined the behaviour of individual ecological metrics across stream types. The number of metrics used varied by stream type: eight metrics for small streams, eleven for medium streams, and seven or eight for large streams depending on subtype (Šporka et al. 2009). Statistically non-significant differences between all sample types were found for three metrics (Percentage of oligosaprobic taxa, Aka+Lit+Psa

taxa, and Rheoindex), and an additional six metrics showed no significant differences when comparing bulk and water samples (e.g., Saprobic Index and Rhithron Type Index). Some of these statistically non-significant differences (e.g., in the case of the Rheoindex) may be explained by the fact that this index was calculated only for certain stream types, resulting in a smaller dataset and consequently lower statistical power to detect differences among sample types. In most cases, however, these non-significant metrics include only those taxa that have appropriate autecological characteristics assigned in the software. Therefore, if a detected taxon lacks a value for saproby, flow preference, feeding functional groups, or preference of microhabitat type in the ASTERICS software, it is excluded from the calculation. For example, information on saprobic valencies is available for only about one-fifth of all taxa included in ASTERICS (Hering et al. 2004). Notably, despite the lower taxonomic detection capacity of water and sediment samples compared to bulk, these sample types may still yield comparable results for several metrics. In contrast to these relatively stable metrics, two indicators consistently showed strong sensitivity to sample type. The most pronounced differences, and the metrics with the strongest influence on the resulting multimetric EQR, were observed for the BMWP index and the number of EPT taxa. These two metrics were applied across all stream types, and their calculation always included the full detected taxonomic spectrum. Regarding EPT, up to 62 unique taxa were revealed in the bulk samples, 40 of which were caddisflies (Trichoptera). In contrast, water and sediment samples together revealed only 6 unique EPT taxa. Macher et al. (2018) compared bulk and water samples and also demonstrated a higher sensitivity of bulk samples in detecting key bioindicator taxa, particularly EPT taxa. These insect groups are generally difficult to identify morphologically, as diagnostic traits are often restricted to specific sexes or life stages (Zhou et al. 2009, 2011). Molecular approaches, on the other hand, provide a more reliable and consistent means of identification. Their integration into biomonitoring programs may reduce the risk of misidentification and improve the consistency of ecological assessments (Haase et al. 2010; Pfrender et al. 2010). Similarly, for the BMWP score, bulk samples detected 18 unique families, the majority of which (11) had relatively high scores (5–10), contributing to a more favourable ecological classification. A similar trend was reported by Fueyo et al. (2024), whose comparison of bulk and water samples from northwestern Spain showed that bulk samples yielded higher IBMWP scores (Iberian BMWP). Given the broader taxonomic coverage enabled by molecular methods, Múrria et al. (2024) recommend recalibrating this metric for DNA-based assessments, as current values may lead to an overestimation of ES. How this can be done was shown in Macher et al. (2025) using the EC Guidance Document No 30 (EC 2015). Our results thus confirm that overall differences between sample types and the resulting ecological quality classification are largely driven by just two metrics, which will clearly require recalibration for future use. This strong influence was further demonstrated by the substantial reduction in EQC differences after excluding these metrics, together with the closely related presence/absence metric Number of Families. As a result, ES classifications became more consistent across sample types, particularly between bulk and water samples.

Methodological limitations and future perspectives

Despite the above findings, which largely favour bulk samples, we recognize that eDNA samples also hold great potential for future applications in the assessment of ES in water bodies. In this context, Múrria et al. (2024) recommend combining bulk and water samples. However, our findings only partially support this recommendation, as we did not observe significant differences when comparing bulk samples with the combined set of water and sediment samples. Environmental samples offer notable advantages, including non-invasiveness, lower labour requirements, and easier standardisation in the field. To improve their future applicability, we suggest further testing of the volume of filtered water. It is expected that filtering larger volumes increases DNA yield. However, this also introduces logistical and mechanical difficulties (Takahashi et al. 2023). About one-third of eDNA studies published between 2012 and 2021 used volumes between 0.5–1 litre, as these volumes are easier to handle in the field and have been shown to capture a representative taxonomic spectrum in both freshwater (Janosik and Johnston 2015; Feng et al. 2020) and marine environments (Andruszkiewicz et al. 2017; Gold et al. 2021). In our case, filtering 1 litre of water did not provide sufficient taxonomic coverage, suggesting that larger volumes may be required, although this warrants further evaluation. This is particularly relevant in situations where low DNA concentrations are expected, such as under specific hydrological or climatic conditions. For sediment samples, the number of replicates may also influence taxon detection. In this study, we collected two sediment samples per site, each with two PCR replicates. However, Wilkinson et al. (2024) recommend using up to six replicates based on species accumulation analyses (Melchior and Baker 2023). Whether increasing the number of sediment replicates to six would significantly affect ES assessment in our context remains unclear and requires further investigation. In addition, the primer used in this study is not optimal for eDNA applications, as BF3/BR2 primers have been shown to perform suboptimally for eDNA due to amplification biases and reduced efficiency for target taxa (Leese et al. 2021). This is particularly relevant for WFD-based biomonitoring, where reliable detection of indicator taxa is essential. Therefore, optimised protocols, including the use of more suitable primers, are required to ensure comparability of results. In the future, however, eDNA samples could potentially serve as a unified source for biomonitoring, enabling the simultaneous assessment of multiple biological quality elements using different primer sets, for example, targeting fish or diatoms. DNA-based monitoring of diatoms has already been successfully tested on a national scale in France (Vasselon et al. 2025).

Conclusions

This study provides a comprehensive assessment comparing three sample types (bulk, water, and sediment) for macrozoobenthos biomonitoring under the WFD using DNA metabarcoding. By applying a broad set of twelve ecological metrics commonly used in multimetric indices across several EU countries, we demonstrate that differences in ES classification are primarily driven by presence/absence metrics, particularly the BMWP index and EPT taxa. In contrast,

abundance-dependent metrics showed comparatively stable responses across sample types, largely reflecting their reliance on a limited subset of taxa with assigned autecological characteristics.

Our results highlight both the opportunities and challenges associated with the implementation of DNA metabarcoding in routine biomonitoring. Bulk samples provided the highest taxonomic coverage and most comprehensive representation of macrozoobenthos, but, similarly to the conventional approach, their invasive nature may limit applicability in sensitive or protected environments. At the same time, the increased detection capacity of bulk samples substantially affects presence/absence metrics, underscoring the need for intercalibration with existing methods to ensure consistent ES assessment. Environmental samples, particularly water and sediment eDNA, offer a non-invasive alternative with clear practical and ethical advantages, especially for long-term monitoring. Optimizing sampling strategies, such as increasing filtered water volumes, the number of sediment replicates, adjusted primers than the one chosen, or PCR replication, may further improve their performance. With appropriate methodological refinements and metric-specific adjustments, DNA metabarcoding has strong potential to support more efficient, standardised, and ecologically sensitive ES assessments under the EU WFD.

Acknowledgements

The authors would like to thank Dr. Mária Šedivá from the Institute of Chemistry, Slovak Academy of Sciences, for her collaboration on NGS sequencing, and Dr. Ondrej Vargovčík for his valuable advice on bioinformatic data processing.

References

Albert JS, Destouni G, Duke-Sylvester SM, Magurran AE, Oberdorff T, Reis RE, Winemiller KO, Ripple WJ (2021) Scientists' warning to humanity on the freshwater biodiversity crisis. Ambio 50: 85–94. https://doi.org/10.1007/s13280-020-01318-8

Allan JD, Castillo MM, Capps KA (2021) Stream ecology. Structure and Function of Running Waters. 3rd edn. Springer International Publishing. https://doi.org/10.1007/978-3-030-61286-3

Andruszkiewicz EA, Starks HA, Chavez FP, Sassoubre LM, Block BA, Boehm AB (2017) Biomonitoring of marine vertebrates in Monterey Bay using eDNA metabarcoding. PLoS ONE 12: e0176343. https://doi.org/10.1371/journal.pone.0176343

Andújar C, Arribas P, Gray C, Bruce C, Woodward G, Yu DW, Vogler AP (2017) Metabarcoding of freshwater invertebrates to detect the effects of a pesticide spill. Molecular Ecology 27: 146–166. https://doi.org/10.1111/mec.14410

Andújar C, Creedy TJ, Arribas P, López H, Salces-Castellano A, Pérez-Delgado AJ, Vogler AP, Emerson BC (2021) Validated removal of nuclear pseudogenes and sequencing artefacts from mitochondrial metabarcode data. Molecular Ecology Resources 21(6): 1772–1787. https://doi.org/10.1111/1755-0998.13337

Armitage PD, Cranston PS, Pinder LCV (1995) The Chironomidae: biology and ecology of non-biting midges. The University of Chicago Press, London, 572 pp. https://doi.org/10.2307/5810

Baena-Bejarano N, Reina C, Martínez-Revelo DE, Medina CA, Tovar E, Uribe-Soto S, Neita-Moreno JC, Gonzalez MA (2023) Taxonomic identification accuracy from BOLD

and GenBank databases using over a thousand insect DNA barcodes from Colombia. PLoS ONE 18(4): e0277379. https://doi.org/10.1371/journal.pone.0277379

Baird DJ, Hajibabaei M (2012) Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. Molecular Ecology 21: 2039–2044. https://doi.org/10.1111/j.1365-294X.2012.05519.x

Beermann AJ, Zizka VMA, Elbrecht V, Baranov V, Leese F (2018) DNA metabarcoding reveals the complex and hidden responses of chironomids to multiple stressors. Environmental Sciences Europe 30: 26. https://doi.org/10.1186/s12302-018-0157-x

Birk S, Bonne W, Borja A, Brucet S, Courrat A, Poikane S, Solimini A, Van De Bund W, Zampoukas N, Hering D (2012) Three hundred ways to assess Europe's surface waters: An almost complete overview of biological methods to implement the Water Framework Directive. Ecological Indicators 18: 31–41. https://doi.org/10.1016/j.ecolind.2011.10.009

Blackman R, Mächler E, Altermatt F, Arnold A, Beja P, Boets P, Egeter B, Elbrecht V, Filipe AF, Jones JI, Macher J, Majaneva M, Martins FMS, Múrria C, Meissner K, Pawlowski J, Schmidt Yáñez PL, Zizka VMA, Leese F, Price B, Deiner K (2019) Advancing the use of molecular methods for routine freshwater macroinvertebrate biomonitoring-the need for calibration experiments. Metabarcoding & Metagenomics 3: e34735. https://doi.org/10.3897/mbmg.3.34735

Bonada N, Prat N, Resh VH, Statzner B (2006) Developments in aquatic insect biomonitoring: A comparative analysis of recent approaches. Annual Review of Entomology 51: 495–523. https://doi.org/10.1146/annurev.ento.51.110104.151124

Brantschen J, Pellissier L, Walser JC, Altermatt F (2022) Evaluation of primer pairs for eDNA-based assessment of Ephemeroptera, Plecoptera, and Trichoptera across a biogeographically diverse region. Environmental DNA 4: 1356–1368. https://doi.org/10.1002/edn3.342

Buchner D, Leese F (2020) BOLDigger - a Python package to identify and organise sequences with the Barcode of Life Data systems. Metabarcoding & Metagenomics 4: e53535. https://doi.org/10.3897/mbmg.4.53535

Buchner D, Beermann AJ, Laini A, Rolauffs P, Vitecek S, Hering D (2019) Analysis of 13,312 benthic invertebrate samples from German streams reveals minor deviations in ecological status class between abundance and presence/absence data. PLoS ONE 14(12): e0226547. https://doi.org/10.1371/journal.pone.0226547

Buss DF, Carlisle DM, Chon TS, Culp J, Harding JS, Keizer-Vlek HE, Robinson WA, Strachan S, Thirion C, Hughes RM (2015) Stream biomonitoring using macroinvertebrates around the globe: a comparison of large-scale programs. Environmental Monitoring and Assessment 187: 4132. https://doi.org/10.1007/s10661-014-4132-8

Chang FH, Lawrence JE, Rios-Touma B, Resh VH (2014) Tolerance values of benthic macroinvertebrates for stream biomonitoring: assessment of assumptions underlying scoring systems worldwide. Environmental monitoring and assessment 186: 2135–2149. https://doi.org/10.1007/s10661-013-3523-6

Chua PY, Bourlat SJ, Ferguson C, Korlevic P, Zhao L, Ekrem T, Meier R, Lawniczak MK (2023) Future of DNA-based insect monitoring. Trends in Genetics 39(7): 531–544. https://doi.org/10.1016/j.tig.2023.02.012

Comtet T, Sandionigi A, Viard F, Casiraghi M (2015) DNA (meta) barcoding of biological invasions: a powerful tool to elucidate invasion processes and help managing aliens. Biological Invasions 17: 905–922. https://doi.org/10.1007/s10530-015-0854-y

Deagle BE, Thomas AC, McInnes JC, Clarke LJ, Vesterinen EJ, Clare EL, Kartzinel TR, Eveson JP (2019) Counting with DNA in metabarcoding studies: How should we

convert sequence reads to dietary data? Molecular Ecology 28(2): 391–406. https://doi.org/10.1111/mec.14734

Derycke S, Maes S, Van den Bulcke L, Vanhollebeke J, Wittoeck J, Hillewaert H, Ampe B, Haegeman A, Hostens K, De Backer A (2021) Detection of Macrobenthos Species With Metabarcoding Is Consistent in Bulk DNA but Dependent on Body Size and Sclerotization in eDNA From the Ethanol Preservative. Frontiers in Marine Science 8: 637858. https://doi.org/10.3389/fmars.2021.637858

Di Muri C, Lawson Handley L, Bean CW, Li J, Peirson G, Sellers GS, Walsh K, Watson HV, Winfield IJ, Hänfling B (2020) Read counts from environmental DNA (eDNA) metabarcoding reflect fish abundance and biomass in drained ponds. Metabarcoding and Metagenomics 4: e56959. https://doi.org/10.3897/mbmg.4.56959

Doi H, Inui R, Akamatsu Y, Kanno K, Yamanaka H, Takahara T, Minamoto T (2017) En-vi-ronmental DNA analysis for estimating the abundance and biomass of stream fish. Freshwater Biology 62: 30–39. https://doi.org/10.1111/fwb.12846

Duarte S, Leite BR, Feio MJ, Costa FO, Filipe AF (2021) Integration of DNA-based approaches in aquatic ecological assessment using benthic macroinvertebrates. Water 3: 331. https://doi.org/10.3390/w13030331

Dudgeon D (2019) Multiple threats imperil freshwater biodiversity in the Anthropocene. Current Biology 29(19): R960–967. https://doi.org/10.1016/j.cub.2019.08.002

Doretto A, Bo T, Bona F, Fenoglio S (2020) Efficiency of Surber net under different substrate and flowconditions: insights for macroinvertebrates sampling and river biomonitoring. Knowledge & Management of Aquatic Ecosystems 421: 10. https://doi.org/10.1051/kmae/2020001

EC (2000) Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy. Official Journal of the European Union L327.

EC (2015) Procedure to Fit New or Updated Classification Methods to the Results of a Completed Intercalibration Exercise. Guidance Document No 30. Publications Office, LU. https://circabc.europa.eu/sd/a/5aee6446-276c-4440-a7de-0d4dec41ed4b/IC_manual_2015_to%20be%20published.pdf [March 24, 2026]

EEA (2024) Europe´s state of water 2024: The need for improved water resilience. European Environment Agency Report 07/2024. Luxembourg: Publications Office of the European Union. https://doi.org/10.4324/9781003392835

Elbrecht V, Leese F (2015) Can DNA-based ecosystem assessments quantify species abundance? Testing primer Bias and biomass-sequence relationships with an innovative metabarcoding protocol. PLoS ONE 10: e0130324. https://doi.org/10.1371/journal.pone.0130324

Elbrecht V, Steinke D (2019) Scaling up DNA metabarcoding for freshwater macrozoobenthos monitoring. Freshwater Biology 64: 380–387. https://doi.org/10.1111/fwb.13220

Elbrecht V, Braukmann TWA, Ivanova NV, Prosser SWJ, Hajibabaei M, Wright M, Zakharov EV, Hebert PDN, Steinke D (2019) Validation of COI metabarcoding primers for terrestrial arthropods. PeerJ 7: e7745 https://doi.org/10.7717/peerj.7745

Elbrecht V, Vamos EE, Meissner K, Aroviita J, Leese F (2017) Assessing strengths and weaknesses of DNA metabarcoding-based macroinvertebrate identification for routine stream monitoring. Methods in Ecology and Evolution 8: 1265–1275. https://doi.org/10.1111/2041-210X.12789

Elbrecht V, Bourlat SJ, Horren T, Lindner A, Mordente A, Noll NW, Schaffler L, Sorg M, Zizka VMA (2021) Pooling size sorted Malaise trap fractions to maximize taxon recovery with metabarcoding. PeerJ 9: e12177. https://doi.org/10.7717/peerj.12177

Erséus C, Gustafsson DR (2009) Cryptic speciation in Clitellate model organism. In: Shain DH (Ed.) Annelids in Modern Biology. John Wiley & Sons, Indianapolis, 31–46. https://doi.org/10.1002/9780470455203.ch3

Feng W, Bulté G, Lougheed SC (2020) Environmental DNA surveys help to identify winter hibernacula of a temperate freshwater turtle. Environmental DNA 2: 200–209. https://doi.org/10.1002/edn3.58

Ferrington LC, Blackwood MA, Wright CA, Crisp NH, Kavanaugh JL, Schmidt FJ (1991) A protocol for using surface-floating pupal exuviae of Chironomidae for rapid bio assessment of changing water quality. Sediment and stream water quality in a changing environment: trends and explanation 203: 181–190.

Ficetola GF, Taberlet P (2023) Towards exhaustive community ecology via DNA metabarcoding. Molecular Ecology 32: 6320–6329. https://doi.org/10.1111/mec.16881

Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental DNA from water samples. Biology Letters 4: 423–425. https://doi.org/10.1098/rsbl.2008.0118

Fonseca VG, Davison PI, Creach V, Stone D, Bass D, Tidbury HJ (2023) The application of eDNA for monitoring aquatic non-indigenous species: practical and policy considerations. Diversity 15: 631. https://doi.org/10.3390/d15050631

Frøslev TG, Kjøller R, Bruun HH, Ejrnæs R, Brunbjerg AK, Pietroni C, Hansen AJ (2017) Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. Nature Communications 8: 1188. https://doi.org/10.1038/s41467-017-01312-x

Frost S, Huni A, Kershaw WE (1971) Evaluation of a kicking technique for sampling stream bottom fauna. Canadian Journal of Zoology 49(2): 167–173. https://doi.org/10.1139/z71-026

Fueyo Á (2024) Assessing DNA metabarcoding and environmental DNA for biomonitoring fluvial ecosystems using macroinvertebrates in peninsular Spain. PhD Thesis, University of Oviedo, Oviedo, Spain. https://doi.org/10.13140/RG.2.2.14159.70568

Fueyo Á, Sánchez O, Coya R, Carleos C, Escudero A, Cordón J, Fernández S, Granero-Castro J, Borrell YJ (2024) The influence of databases enrichment using local macroinvertebrate genetic references for metabarcoding based biodiversity studies in river monitoring. Ecological Indicators 158: 111454. https://doi.org/10.1016/j.ecolind.2023.111454

Fueyo Á, Sánchez O, Carleos C, Granero-Castro J, Borrell YJ (2025) Technical challenges in the implementation of macroinvertebrate DNA metabarcoding in river biomonitoring: A case study from peninsular Spain - Advantages, limitations and roadmap. Ecological Solutions and Evidence 6: e70125. https://doi.org/10.1002/2688-8319.70125

Furse M, Hering D, Moog O, Verdonschot PFM, Johnson RK, Brabec K, Gritzalis K, Buffagni A, Pinto P, Friberg N, Murray-Bligh J, Kokes J, Alber R, Usseglio-Polatera P, Haase P, Sweeting R, Bis B, Szoszkiewicz K, Soszka H, Springe G, Sporka F, Krno I (2006) The STAR project: Content, objectives and approaches. Hydrobiologia 566: 3–29. https://doi.org/10.1007/s10750-006-0067-6

Giraudoux P (2023) *pgirmess*: Spatial Analysis and Data Mining for Field Ecologists. R package version 2.0.2. https://doi.org/10.32614/cran.package.pgirmess

Gleason JE, Elbrecht V, Braukmann TWA, Hanner RH, Cottenie K (2021) Assessment of stream macroinvertebrate communities with eDNA is not congruent with tissue-based metabarcoding. Molecular Ecology 30: 3239–3251. https://doi.org/10.1111/mec.15597

Gleason JE, Hanner RH, Cottenie K (2023) Hidden diversity: DNA metabarcoding reveals hyper-diverse benthic invertebrate communities. BMC Ecology and Evolution 23: 19. https://doi.org/10.1186/s12862-023-02118-w

Gold Z, Sprague J, Kushner DJ, Zerecero Marin E, Barber PH (2021) eDNA metabarcoding as a biomonitoring tool for marine protected areas. PLoS ONE 16: e0238557. https://doi.org/10.1371/journal.pone.0238557

Haase P, Murray-Bligh J, Lohse S, Pauls S, Sundermann A, Gunn R, Clarke R (2006) Assessing the impact of errors in sorting and identifying macroinvertebrate samples. Hydrobiologia 566: 505–521. https://doi.org/10.1007/s10750-006-0075-6

Haase P, Pauls SU, Schindehu K, Sundermann A (2010) First audit of macroinvertebrate samples from an EU Water Framework Directive monitoring program: human error greatly lowers precision of assessment results. Journal of the North American Benthological Society 29(4): 1279–1291. https://doi.org/10.1899/09-183.1

Hajibabaei M, Porter TM, Robinson CV, Baird DJ, Shokralla S, Wright MT (2019) Watered-down biodiversity? A comparison of metabarcoding results from DNA extracted from matched water and bulk tissue biomonitoring samples. PloS ONE 14: e0225409. https://doi.org/10.1371/journal.pone.0225409

Hending D (2025) Cryptic species conservation: a review. Biological Reviews 100(1): 258–274. https://doi.org/10.1111/brv.13139

Hering D, Borja A, Carstensen J, Carvalho L, Elliott M, Feld CK, Heiskanen A-S, Johnson RK, Moe J, Pont D, Solheim AL, Van de Bund W (2010) The European Water Framework Directive at the age of 10: a critical review of the achievements with recommendations for the future. Science of the total Environment 408(19): 4007–4019. https://doi.org/10.1016/j.scitotenv.2010.05.031

Hering D, Moog O, Sandin L, Verdonschot PFM (2004) Overview and application of the AQEM assessment system. Hydrobiologia 516: 1–20. https://doi.org/10.1023/B:HYDR.0000025255.70009.a5

Hodkinson ID, Jackson JK (2005) Terrestrial and aquatic invertebrates as bioindicators for environmental monitoring, with particular reference to mountain ecosystems. Environmental management 35: 649–666. https://doi.org/10.1007/s00267-004-0211-x

Janosik AM, Johnston CE (2015) Environmental DNA as an effective tool for detection of imperiled fishes. Environmental Biology of Fishes 98: 1889–1893. https://doi.org/10.1007/s10641-015-0405-5

Ji F, Han D, Yan L, Yan S, Zha J, Shen J (2022) Assessment of benthic invertebrate diversity and river ecological status along an urbanized gradient using environmental DNA metabarcoding and a traditional survey method. Science of the Total Environment 806: 150587. https://doi.org/10.1016/j.scitotenv.2021.150587

Joseph C, Faiq ME, Li Z, Chen G (2022) Persistence and degradation dynamics of eDNA affected by environmental factors in aquatic ecosystems. Hydrobiologia 849: 4119–4133. https://doi.org/10.1007/s10750-022-04959-w

Kampstra P (2008) "Beanplot: A Boxplot Alternative for Visual Comparison of Distributions." Journal of Statistical Software, Code Snippets 28(1): 1–9. https://doi.org/10.18637/jss.v028.c01

Kirse A, Bourlat SJ, Langen K, Zapke B, Zizka VMA (2022) Comparison of destructive and non-destructive DNA extraction methods for the metabarcoding of arthropod bulk samples. Molecular Ecology Resources 23: 92–105. https://doi.org/10.1111/1755-0998.13694

Klymus KE, Marshall NT, Stepien CA (2017) Environmental DNA (eDNA) metabarcoding assays to detect invasive invertebrate species in the Great Lakes. PLoS ONE 12: e0177643. https://doi.org/10.1371/journal.pone.0177643

Krehenwinkel H, Wolf M, Lim JY, Rominger AJ, Simison WB, Gillespie RG (2017) Esti-mating and mitigating amplification bias in qualitative and quantitative arthropod metabarcoding. Scientific Reports 7: 17668. https://doi.org/10.1038/s41598-017-17333-x

Leese F, Sander M, Buchner D, Elbrecht V, Haase P, Zizka VMA (2021) Improved freshwater macroinvertebrate detection from environmental DNA through minimized nontarget amplification. Environmental DNA 3: 261–276. https://doi.org/10.1002/edn3.177

Liu M, Clarke LJ, Baker SC, Jordan GJ, Burridge CP (2019) A practical guide to DNA metabarcoding for entomological ecologists. Ecological Entomology 45: 373–385. https://doi.org/10.1111/een.12831

Löbl I, Klausnitzer B, Hartmann M, Krell FT (2023) The Silent Extinction of Species and Taxonomists - An Appeal to Science Policymakers and Legislators. Diversity 15(10): 1053. https://doi.org/10.3390/d15101053

Macher JN, Vivancos A, Piggott JJ, Centeno FC, Matthaei CD, Leese F (2018) Comparison of environmental DNA and bulk sample metabarcoding using highly degenerate cytochrome c oxidase I primers. Molecular Ecology Resources 18: 1456–1468. https://doi.org/10.1111/1755-0998.12940

Macher TH, Beermann AJ, Leese F (2021) TaxonTableTools: A comprehensive, platform-independent graphical user interface software to explore and visualise DNA metabarcoding data. Molecular Ecology Resources 21: 1705–1714. https://doi.org/10.1111/1755-0998.13358

Macher TH, Beermann AJ, Arle J, Foerster J, Greyer M, Mora D, Koschorreck J, Rolauffs P, Rother A, Schüller S, Zimmermann J, Hering D, Leese F (2025) Fit for purpose? Evaluating benthic invertebrate DNA metabarcoding for ecological status class assessment in streams under the Water Framework Directive. Water Research 272: 122987. https://doi.org/10.1016/j.watres.2024.122987

Macko P, Derka T, Čiamporová-Zaťovičová Z, Grabowski M, Čiampor Jr F (2024) Detailed DNA barcoding of mayfies in a small European country proved how far we are from having comprehensive barcode reference libraries. Molecular Ecology Resources 24: e13954. https://doi.org/10.1111/1755-0998.13954

Macko P, Čiampor Jr F, Šamulková M, Vargovčík O, Tuhrinová K, Čiamporová-Zaťovičová Z (2025) High Altitudes as Reservoirs of Unique Genetic Diversity: A Case Study on Aquatic Beetles in Glacial Lakes of Tatra Mountains. Freshwater Biology 70(8): e70083. https://doi.org/10.1111/fwb.70083

Majaneva M, Sundt-Hansen LE, Brandsegg H, Sivertsgård R, Bongard T, Fossøy F (2024) Comparing methods and indices for biodiversity and status assessment in a hydropower-regulated river. Hydrobiologia 851: 2139–2157. https://doi.org/10.1007/s10750-023-05448-4

Marshall JC, Steward AL, Harch BD (2006) Taxonomic resolution and quantification of freshwater macroinvertebrate samples from an Australian dryland river: the benefits and costs of using species abundance data. Hydrobiologia 572: 171–194. https://doi.org/10.1007/s10750-005-9007-0

Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet Journal 17(1): 10–12. https://doi.org/10.14806/ej.17.1.200

Melchior M, Baker C (2023) Environmental DNA guidelines and field protocols for lotic systems. NIWA Taihoro Nukurangi, 84 pp.

Morinière J, Hendrich L, Balke M, Beermann AJ, König T, Hess M, Koch S, Müller R, Leese F, Hebert PDN, Hausmann A, Schubart ChD, Haszprunar G (2017) A DNA barcode library for Germany's mayfies, stonefies and caddisfies (Ephemeroptera, Plecoptera and Trichoptera). Molecular Ecology Resources 17(6): 1293–1307. https://doi.org/10.1111/1755-0998.12683

Mrozińska N, Obolewski K (2024) Morphological taxonomy and DNA barcoding: Should they be integrated to improve the identification of chironomid larvae (Diptera)? Ecohydrology & Hydrobiology 24(1): 1–10. https://doi.org/10.1016/j.ecohyd.2023.11.007

Múrria C, Wangensteen OS, Somma S, Väisänen L, Fortuño P, Arnedo MA, Prat N (2024) Taxonomic accuracy and complementarity between bulk and eDNA metabarcoding provides an alternative to morphology for biological assessment of freshwater macroinvertebrates. Science of The Total Environment 935: 173243. https://doi.org/10.1016/j.scitotenv.2024.173243

Pawlowski J, Bruce K, Panksep K, Aguirre FI, Amalfitano A, Apothéloz-Perret-Gentil L, Baussant T, Bouchez A, Carugati L, Cermakova K, Cordier T, Corinaldesi C, Costa FO, Danovaro R, Dell'Anno A, Duarte S, Eisendle U, Ferrari BJD, Frontalini F, Frühe L, Haegerbaeumer A, Kisand V, Krolicka A, Lanzén A, Leese F, Lejzerowicz F, Lyautey E, Maček I, Sagova-Marečková M, Pearman JK, Pochon X, Stoeck T, Vivien R, Weigand A, Fazi S (2022) Environmental DNA metabarcoding for benthic monitoring: A review of sediment sampling and DNA extraction methods. Science of The Total Environment 818: 151783. https://doi.org/10.1016/j.scitotenv.2021.151783

Pfenninger M, Nowak C, Kley C, Steinke D, Streit B (2007) Utility of DNA taxonomy and barcoding for the inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species. Molecular Ecology 16: 1957–1968. https://doi.org/10.1111/j.1365-294X.2006.03136.x

Pfrender M, Hawkins C, Bagley M, Courtney G, Creutzburg B, Epler J, Fend S, Ferrington Jr LC, Hartzell PL, Jackson S, Larsen DP, Lévesque CA, Morse JC, Petersen MJ, Ruiter D, Schindel D, Whiting M (2010) Assessing macroinvertebrate biodiversity in freshwater ecosystems: Advances and challenges in DNA-basedapproaches. The Quarterly Review of Biology 85(3): 319–340. https://doi.org/10.1086/655118

Pinder LCV (1986) Biology of fresh-water Chironomidae. Annual Review of Entomology 31: 1–23. https://doi.org/10.1146/annurev.en.31.010186.000245

R Core Development Team (2022) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://doi.org/10.32614/r.manuals

Ratnasingham S, Hebert PD (2007) BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Molecular Ecology Notes 7: 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Raupach MJ, Rulik B, Spelda J (2022) Surprisingly high geneticdivergence of the mitochondrial DNA barcode fragment (COI) within Central European woodlice species (Crustacea, Isopoda, Oniscidea). ZooKeys 1082: 103–125. https://doi.org/10.3897/zookeys.1082.69851

Reid AJ, Carlson AK, Creed IF, Eliason EJ, Gell PA, Johnson PTJ, Kidd KA, MacCormack TJ, Olden JD, Ormerod SJ, Smol JP, Taylor WW, Tockner K, Vermaire JC, Dudgeon D, Cooke SJ (2019) Emerging threats and persistent conservation challenges for freshwater biodiversity. Biological Reviews 94: 849–873. https://doi.org/10.1111/brv.12480

Rivera SF, Vasselon V, Mary N, Monnier O, Rimet F, Bouchez A (2021) Exploring the capacity of aquatic biofilms to act as environmental DNA samplers: test on macroinvertebrate communities in rivers. Science of the Total Environment 763: 144208. https://doi.org/10.1016/j.scitotenv.2020.144208

Rognes T, Flouri T, Nichols B, Quince C, Mahé F (2016) VSEARCH: A versatile open source tool for metagenomics. PeerJ 4: e2584. https://doi.org/10.7717/peerj.2584

Rosenberg DM, Resh VH (1993) Freshwater biomonitoring and benthic invertebrates. Springer, New York, 488 pp.

Roslin T, Somervuo P, Pentinsaari M, Hebert PD, Agda J, Ahlroth P, Anttonen P, Aspi J, Blagoev G, Blanco S, Chan D, Clayhills T, deWaard J, deWaard S, Elliot T, Elo R, Haapala S, Helve E, Ilmonen J, Hirvonen P, Ho C, Itämies J, Ivanov V, Jakovlev J, Juslén A, Jussila R, Kahanpää J, Kaila L, Kaitila JP, Kakko A, Kakko I, Karhu A, Karjalainen S, Kjaerandsen J, Koskinen J, Laasonen EM, Laasonen L, Laine E, Lampila P, Levesque-Beaudin V, Lu L, Lähteenaro M, Majuri P, Malmberg S, Manjunath R, Martikainen P, Mattila J, McKeown J, Metsälä P, Miklasevskaja M, Miller M, Miskie R, Muinonen A, Mukkala VM, Naik S, Nikolova N, Nupponen K, Ovaskainen O, Österblad I, Paasivirta L, Pajunen T, Parkko P, Paukkunen J, Penttinen R, Perez K, Pohjoismäki J, Prosser S, Raekunnas M, Rahulan M, Rannisto M, Ratnasingham S, Raukko P, Rinne A, Rintala T, Romo SM, Salmela J, Salokannel J, Savolainen R, Schulman L, Sihvonen P, Soliman D, Sones J, Steinke C, Ståhls G, Tabell J, Tiusanen M, Várkonyi G, Vesterinen EJ, Viitanen E, Vikberg V, Viitasaari M, Vilen J, Warne C, We C, Winqvist K, Zakharov E, Mutanen M (2022) A molecular-based identifcation resource for the arthropods of Finland. Molecular Ecology Resources 22(2): 803–822. https://doi.org/10.1111/1755-0998.13510

Salis R, Sunde J, Gubonin N, Franzén M, Forsman A (2024) Performance of DNA metabarcoding, standard barcoding and morphological approaches in the identification of insect biodiversity. Molecular Ecology Resources 24(8): e14018. https://doi.org/10.1111/1755-0998.14018

Šamulková M, Čiamporová-Zaťovičová Z, Čiampor F, Tuhrinová K, Macko P (2025a) Is the DNA barcode database fit for purpose? Assessing the feasibility of reverse taxonomy in five key aquatic insect orders relevant to freshwater biomonitoring under the Water Framework Directive. Biologia 80: 2733–2742. https://doi.org/10.1007/s11756-025-01999-0

Šamulková M, Beracko P, Macko P, Vargovčík O, Tuhrinová K, Čiamporová-Zaťovičová Z, Lešťáková M, Mišíková Elexová E, Čiampor F (2025b) Evaluating the potential of DNA metabarcoding for ecological status assessment under the Water Framework Directive: A case study on benthic invertebrates from Western Carpathian streams. Metabarcoding and Metagenomics 9: e163640. https://doi.org/10.3897/mbmg.9.163640

Sander M, Beerman AJ, Buchner D, Weiss M, Werner MT, Leese F (2025a) Capture—Incubate—Release: An Animal-Friendly Approach to Assess Local Aquatic Macroinvertebrate Species Diversity Through Environmental DNA Metabarcoding. Environmental DNA 7: e70112. https://doi.org/10.1002/edn3.70112

Sander M, Beermann AJ, Brömmling D, Buchner D, Weiss M, Leese F (2025b) Comparison of three strategies for local bioassessments in streams using environmental DNA and RNA metabarcoding of macroinvertebrates. Metabarcoding and Metagenomics 9: e148923. https://doi.org/10.3897/mbmg.9.148923

Schultz JA, Hebert PDN (2022) Do pseudogenes pose a problem for metabarcoding marine animal communities? Molecular Ecology Resources 22(8): 2897–2914. https://doi.org/10.1111/1755-0998.13667

Shelton AO, Gold ZJ, Jensen AJ, D'Agnese E, Andruszkiewicz AE, van Cise A, Gallego R, Ramón-Laca A, Garber-Yonts M, Parsons K, Kelly RP (2023) Toward quantitative me-tabarcoding. Ecology 104(2): e3906. https://doi.org/10.1002/ecy.3906

Šporka F, Pastuchová Z, Hamerlík L, Dobiášová M, Beracko P (2009) Assessment of running waters (Slovakia) using benthic macroinvertebrates – derivation of ecologi-

cal quality classes with respect to altitudinal gradients. Biologia 64(6): 1196–1205. https://doi.org/10.2478/s11756-009-0201-9

Szucsich NU (2015) ABOL–DNA-Barcoding als Impuls für die Biodiversitätsforschung in Österreich. Acta ZooBot Austria 152: 157–160.

Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH (2012) Environmental DNA. Molecular Ecology 21: 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Takahashi M, Saccò M, Kestel JH, Nester G, Campbell MA, Van der Heyde M, Heydenrych MJ, Juszkiewicz DJ, Nevill P, Dawkins KL, Bessey C, Fernandes K, Miller H, Power M, Mousavi-Derazmahalleh M, Newton JP, White NE, Richards ZT, Allentoft ME (2023) Aquatic environmental DNA: A review of the macro-organismal biomonitoring revolution. Science of The Total Environment 873: 162322. https://doi.org/10.1016/j.scitotenv.2023.162322

Turner CR, Uy KL, Everhart RC (2015) Fish environmental DNA is more concentrated in aquatic sediments than surface water. Biological Conservation 183: 93–102. https://doi.org/10.1016/j.biocon.2014.11.017

V. den Bulcke L, De Backer A, Hillewaert H, Maes S, Seghers S, Waegeman W, Wittoeck J, Hostens K, Derycke S (2024) Comparative study of traditional and DNA-based methods for environmental impact assessment: A case study of marine aggregate extraction in the North Sea. Science of the Total Environment 946: 174106. https://doi.org/10.1016/j.scitotenv.2024.174106

van der Lee GH, Polling M, Laros I, Verdonschot RCM (2026) Cross-comparing different protocols for DNA metabarcoding of freshwater macroinvertebrates. Hydrobiologia 853: 1217–1231. https://doi.org/10.1007/s10750-025-05993-0

Vamos E, Elbrecht V, Leese F (2017) Short COI markers for freshwater macroinvertebrate metabarcoding. Metabarcoding and Metagenomics 1: e14625. https://doi.org/10.3897/mbmg.1.14625

Vargovčík O, Čiamporová-Zaťovičová Z, Beracko P, Kopáček J, Macko P, Tuhrinová K, Čiampor Jr F (2024) Environmental gradients and optimal fixation time revealed with DNA metabarcoding of benthic sample fixative. Scientific reports 14: 18396. https://doi.org/10.1038/s41598-024-68939-x

Vasselon V, Rivera SF, Ács É, Almeida SFP, Andree KB, Apothéloz-Perret-Gentil L, Bailet B, Baričević A, Beentjes KK, Bettig J, Bouchez A, Capelli C, Chardon C, Duleba M, Elersek T, Genthon C, Jablonska M, Jacas L, Kahlert M, Kelly MG, Macher JN, Mauri F, Moletta-Denat M, Mortágua A, Pawlowski J, Pérez-Burillo J, Pfannkuchen M, Pilgrim E, Pissaridou P, Rimet F, Stanic K, Tapolczai K, Theroux S, Trobajo R, Van der Hoorn B, Vasquez MI, Vidal M, Wanless D, Warren J, Zimmermann J, Paix B (2025) Proficiency testing and cross-laboratory method comparison to support standardisation of diatom DNA metabarcoding for freshwater biomonitoring. Metabarcoding and Metagenomics 9: e133264. https://doi.org/10.3897/mbmg.9.133264

Vivien R, Wyler S, Lafont M, Pawlowski J (2015) Molecular barcoding of aquatic oligochaetes: implications for biomonitoring. PLoS ONE 10: e0125485. https://doi.org/10.1371/journal.pone.0125485

Vivien R, Apothéloz-Perret-Gentil L, Pawlowski J, Werner I, Lafont M, Ferrari BJD (2020) High-throughput DNA barcoding of oligochaetes for abundance-based indices to assess the biological quality of sediments in streams and lakes. Scientific Reports 10: 2041. https://doi.org/10.1038/s41598-020-58703-2

Vourka A, Karaouzas I, Parmakelis A (2023) River benthic macroinvertebrates and environmental DNA metabarcoding: a scoping review of eDNA sampling, extraction, amplification and sequencing methods. Biodiversity and Conservation 32: 4221–4238. https://doi.org/10.1007/s10531-023-02710-y

Wang Y, Chen K, Gao J, Wang M, Dong J, Xie Y, Giesy JP, Jin X, Wang B (2021) Environmental DNA of preservative ethanol performed better than water samples in detecting macroinvertebrate diversity using metabarcoding. Diversity and Distribution 27: 1989–2002. https://doi.org/10.1111/ddi.13284

Wheeler QD, Raven PH, Wilson EO (2004) Taxonomy: Impediment or Expedient? Science 303: 285–285. https://doi.org/10.1126/science.303.5656.285

Wilkinson SP, Gault AA, Welsh SA, Smith JP, David BO, Hicks AS, Fake DR, Suren AM, Shaffer MR, Jarman SN, Bunce M (2024) TICI: a taxon-independent community index for eDNA-based ecological health assessment. PeerJ 12: e16963. https://doi.org/10.7717/peerj.16963

Zhang J, Kobert K, Flouri T, Stamatakis A (2013) PEAR: A fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30(5): 614–620. https://doi.org/10.1093/bioinformatics/btt593

Zhang H, Bu W (2022) Exploring large-scale patterns of genetic vari-ation in the COI gene among Insecta: Implications for DNA barcod-ing and threshold-based species delimitation studies. Insects 13(5): 425. https://doi.org/10.3390/insects13050425

Zhou X, Adamowicz SJ, Jacobus LM, DeWalt RE, Hebert PDN (2009) Towards a comprehensive barcode library for arctic life - Ephemeroptera, Plecoptera, and Trichoptera of Churchill, Manitoba, Canada. Frontiers in Zoology 6: 30. https://doi.org/10.1186/1742-9994-6-30

Zhou X, Robinson JL, Geraci CJ, Parker CR, Flint Jr OS, Etnier DA, Ruiter D, DeWalt RE, Jacubos LM, Hebert PDN (2011) Accelerated construction of aregional DNA-barcode reference library: Caddisflies (Trichoptera) inthe Great Smoky Mountains National Park. Journal of the North American Benthological Society 30(1): 131–162. https://doi.org/10.1899/10-010.1

Supplementary material 1

List of sampling sites and their detailed characteristics processed using DNA metabarcoding in this study

Authors: Michaela Šamulková, Pavel Beracko, Patrik Macko, Kornélia Tuhrinová, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: xlsx

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.10.185694.suppl1

Supplementary material 2

List of species (DNA dataset) obtained in this study using the DNA metabarcoding approach after merging replicates in TTT

Authors: Michaela Šamulková, Pavel Beracko, Patrik Macko, Kornélia Tuhrinová, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: xlsx

Explanation note: The dataset includes results for individual sampling sites and sample types, with species presence indicated by read counts and absence marked as 0.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.10.185694.suppl2

Supplementary material 3

List of metrics used to calculate the Multimetric Index for ten different stream types

Authors: Michaela Šamulková, Pavel Beracko, Patrik Macko, Kornélia Tuhrinová, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: xlsx

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.10.185694.suppl3

Supplementary material 4

Reference values for metrics used in partial EQR (Ecological Quality Ratio) calculations by stream type

Authors: Michaela Šamulková, Pavel Beracko, Patrik Macko, Kornélia Tuhrinová, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: xlsx

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.10.185694.suppl4

Supplementary material 5

Graphical representation of the number of detected taxa across different taxonomic levels (species, genus, family, order) for three datasets (OTUs, Linnaean species, and ASTERICS) and sample types (bulk, water, sediment)

Authors: Michaela Šamulková, Pavel Beracko, Patrik Macko, Kornélia Tuhrinová, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: tiff

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.10.185694.suppl5

Supplementary material 6

Sequencing depth across sample types (bulk, water, and sediment eDNA), expressed as log-transformed read counts per locality

Authors: Michaela Šamulková, Pavel Beracko, Patrik Macko, Kornélia Tuhrinová, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: tiff

Explanation note: Box boundaries represent the 25th and 75th percentiles, the central line indicates the median, and points represent individual site values.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.10.185694.suppl6

Supplementary material 7

Values of individual metrics from ASTERICS and EQR calculations for ecological status assessment

Authors: Michaela Šamulková, Pavel Beracko, Patrik Macko, Kornélia Tuhrinová, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: xlsx

Explanation note: Values of individual metrics from ASTERICS and EQR calculations for ecological status assessment: individual sheets for each stream type include metric values (A.) and calculated partial EQRs (B.); additional sheets summarize all partial EQRs, final multimetric EQRs, and classification into five ecological quality classes.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.10.185694.suppl7

Supplementary material 8

Additional information

Authors: Michaela Šamulková, Pavel Beracko, Patrik Macko, Kornélia Tuhrinová, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: zip

Explanation note: **8a**. List of all detected EPT taxa (Ephemeroptera, Plecoptera, Trichoptera), including their occurrence across individual sample types (bulk, water, and sediment) (Supplementary Material 8a.xlsx). **8b.** List of all families contributing to the BMWP score and Number of Families metrics, including their presence across sample types (bulk, water, and sediment) and corresponding BMWP scores.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.10.185694.suppl8

Supplementary material 9

Boxplots showing differences in EQR values between sample types, including results of Tukey HSD post-hoc tests. EQR values were calculated excluding metrics EPT taxa, BMWP score, and Number of Families

Authors: Michaela Šamulková, Pavel Beracko, Patrik Macko, Kornélia Tuhrinová, Zuzana Čiamporová-Zaťovičová, Fedor Čiampor Jr

Data type: tiff

Explanation note: Box boundaries represent the 25th and 75th percentiles, the central line indicates the median, and points represent individual EQR values for each site. Coloured background bands indicate ecological quality classes (EQC): red – bad, orange – poor, yellow – moderate, green – good, and blue – very good. The figure also includes a table summarising results of parametric (one-way ANOVA) and non-parametric (Kruskal–Wallis) tests comparing EQR values among sample types for the multimetric index as well as for individual metrics. The ANOVA section includes degrees of freedom (between-group and within-group), F-statistic, and p-values.

Copyright notice: This dataset is made available under the Open Database License (http://opendatacommons.org/licenses/odbl/1.0/). The Open Database License (ODbL) is a license agreement intended to allow users to freely share, modify, and use this Dataset while maintaining this same freedom for others, provided that the original source and author(s) are credited.

Link: https://doi.org/10.3897/mbmg.10.185694.suppl9

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.
No AI tools were used in the preparation of this manuscript.

Funding

This work was financially supported by the Slovak National Grant Agency (VEGA), project no. 2/0084/21 and 1/0170/25.

Author contributions

Conceptualization: MŠ, PB, PM, FČ. Data curation: MŠ. Resources: MŠ, PM, ZČZ, FČ. Formal analysis: PB. Methodology: MŠ, PM, FČ. Software: MŠ, PM. Investigation: MŠ, PM, KT. Funding acquisition: ZČZ. Supervision: FČ. Validation: FČ. Visualization: MŠ, PM. Writing – original draft: MŠ, PB. Writing – review, & editing: PM, KT, ZČZ, FČ.

Author ORCIDs

M. Šamulková https://orcid.org/0009-0000-9599-5255
P. Beracko https://orcid.org/0000-0001-7680-0854
P. Macko https://orcid.org/0009-0008-0714-7490
K. Tuhrinová https://orcid.org/0009-0007-2315-3826
Z. Čiamporová-Zaťovičová https://orcid.org/0000-0003-0506-6212
F. Čiampor Jr https://orcid.org/0000-0001-6269-3592

Data availability

Additional data are in Suppl. materials on FigShare (https://figshare.com/) under https://doi.org/10.6084/m9.figshare.29092283 or available on request. The sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1442384. The data will be made publicly available upon publication of the manuscript.